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Large scale parallel pyrosequencing technology: PRRSV strain VR-2332 nsp2 deletion mutant stability in swine☆

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ABSTRACT

Fifteen porcine reproductive and respiratory syndrome virus (PRRSV) isolate genomes were derived simultaneously using 454 pyrosequencing technology. The viral isolates sequenced were from a recent swine study, in which engineered Type 2 prototype PRRSV strain VR-2332 mutants, with 87, 184, 200, and 403 amino acid deletions in the second hypervariable region of nsp2, were found to be stable in the nsp2 coding region after in vivo infection (Faaberg et al., 2010). Furthermore, 3 of 4 mutants achieved replication kinetics similar to *wt* virus by study end. We hypothesized that other mutations elsewhere in the virus may have contributed to their replication fitness in swine. To further assess the stability of the engineered viruses, all sequenced genomes were compared and contrasted. No specific mutations occurred in all nsp2 deletion mutant genomes that were not also seen in the parent genome of Type 2 PRRSV strain VR-2332. Second site (non-nsp2) deletions and/or insertions were not evident after replication in swine. The number of point mutations seen increased slightly with deletion size, but even the largest deletion (403 aa) had very few consensus mutations. Thus, our findings provide further substantiation that the nsp2 deletion mutant genomes were genetically stable after in vivo passage.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae* of the order *Nidovirales* (Cavanagh, 1997). The single-stranded, positive-sense RNA virus genome varies in length from 15 to 15.5 kilobases, and consists of a 5′ untranslated region (UTR) immediately followed by a long open reading frame (ORF1) that codes for the replicase polyprotein (1a and 1ab), followed by several overlapping ORFs coding for structural proteins, and a 3′ UTR. The viral replicase polyprotein is processed cotranslationally into at least 14 proteins by self-encoded papain-like cysteine proteases [PLP1α and 1β in nonstructural protein (nsp) 1, and PLP2 in nsp2] and a serine protease in nsp4. PLP2 (also known as PL2 or cysteine protease) belongs to the superfamily of proteases homologous to the *Ovarian tumor* (OTU) gene product of *Drosophila* (Frias-Staheli et al., 2007; Makarova et al., 2000; Sun et al., 2010). Consistent with other viral OTU homologs, the PLP2 protease of Type 1 PRRSV has been shown to possess deubiquitinating activity in vitro (Frias-Staheli et al., 2007; Makarova et al., 2000; Sun et al., 2010). Nsp2 harbors the PLP2 domain near the N-terminal end, flanked by two regions shown to be hypervariable among PRRSV strains (Han et al., 2006), followed by 3–4 transmembrane domains and by a relatively conserved C-terminal region. The variation in PRRSV strain genome size has been shown to be largely due to deletions or insertions in the second hypervariable (HV2) domain of nsp2 (Han et al., 2006). Several engineered genomes have used nsp2 deletions and/or insertions in this same region to investigate viral protein function and produce marker vaccines (Chen et al., 2010; Fang et al., 2008; Kim et al., 2007, 2009; Zhou et al., 2009). Our laboratory has produced several nsp2 deletion constructs in the prototype Type 2 strain VR-2332, and has inserted foreign tags to investigate the actions of the PLP2 protease in vitro (Han et al., 2007, 2009, 2010). Recently, we also investigated the stability and replication kinetics as well as selected immune parameters after infection of swine with four of these deletion mutants, coding for deletions in nsp2 varying in size from 87 to 403 amino acids (Faaberg et al., 2010). We reported that the deletion mutants initially had diminished capacities to replicate in swine, but all but the large deletion virus (403 amino acids removed from nsp2) recovered to parental viral RNA levels by study end. The prior study also showed that the ORF1 region coding for nsp2 (3688

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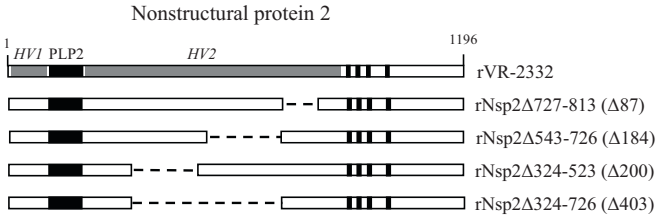

Fig. 1. PRRSV nsp2 deletion mutant schematic. Treatment groups used for deletion mutant sequencing study. Abbreviations: hypervariable regions of nsp2 (HV1 and HV2), defined previously, are shown in solid grey (Han et al., 2006), chymotrypsin-like cysteine protease (PLP2), predicted nsp2 transmembrane regions are indicated by short black bars (▮).

bases for strain VR-2332) was genetically stable in all four mutants (Faaberg et al., 2010). Our hypothesis was that other regions of the PRRSV deletion mutant genomes possessed alterations/adaptations that led to the recovery to *wt* virus replication levels for 3 of the 4 nsp2 deleted viruses. In order to understand the effects of the nsp2 deletions on the genetic stability of other areas of the virus genome and to probe reasons for viral fitness, a protocol was devised and implemented to acquire several PRRSV genome sequences in one sequencing effort.

We now report the successful simultaneous 454 pyrosequencing of 15 complete or nearly complete genomes of the engineered viruses after passage in swine. The complete sequences were then compared and contrasted to understand the stability of engineered mutant genomes. We found that no identical mutations occurred in all nsp2 deletion mutant genomes that were not seen in the recombinant wild-type (*wt*) VR-2332 genome. Second site (non-nsp2) deletions and/or insertions were not detected. In general, the number of mutations seen increased with deletion size, but even the largest deletion (403 aa) had very few consensus mutations. Thus, the nsp2 deletion mutant genomes were genetically stable in vivo.

2. Materials and methods

2.1. Virus

Recombinant viral strain VR-2332 (rV7; *wt*) and four VR-2332 nsp2 deletion mutants (rΔ727-813, rΔ543-726, rΔ324-523, and rΔ324-726) have been described previously (Fig. 1) (Han et al., 2007). The engineered mutants correspond to deletion sizes of 87 (Δ87), 184 (Δ184), 200 (Δ200) and 403 (Δ403) amino acids, respectively. In a previous study, all five viruses had been individually inoculated into groups of four young pigs at 4 weeks of age (Faaberg et al., 2010).

2.2. Swine study

In the present animal experiment, 18 conventionally raised 3-week-old weaned pigs were purchased from a PRRSV-negative herd. Upon delivery to the National Animal Disease Center (NADC), pigs were randomly allocated to one of three treatment groups ($n = 6$/group) housed separately in isolation facilities following the NADC Animal Care and Use Committee guidelines. Each group was acclimated for 1 week prior to the beginning of the study (Day 0), at which time all pigs within the group were inoculated with one of 3 virus preparations. Groups 1 and 2 received the wild type (*wt*) or large deletion (Δ403) recombinant viruses, respectively, that were used in a previous in vivo study (Faaberg et al., 2010). Pigs were inoculated with an intramuscular injection at one site with 2 ml (2×10^3 TCID/ml) of cell culture propagated virus. In order to examine the growth kinetics and stability of the large deletion virus after an additional in vivo passage, pigs in group 3 were inoculated intramuscularly with lung lavage fluid (2 ml at each of 2 sites) collected from one pig 35 days post-challenge with the cell-culture propagated Δ403 virus (Faaberg et al., 2010). The lung lavage fluid had been negative by virus isolation, but positive by PRRSV qRT PCR for Δ403 (rΔ324-726) RNA. Serum was collected from all pigs on days 0, 2, 4, 7, 9, 11, 15, 21, 28, and 35 post-inoculation. On day 35, each pig was euthanized and necropsied to collect lung lavage fluid. The weight of the tracheobronchial lymph node located at the base of the left bronchi (LNW) was compared to the body weight (BW) to provide a clinical assessment of relative PRRSV pathogenicity. Because the purpose of the study was to examine the effect of swine passage on the replication kinetics of the large deletion virus, no negative control animals were utilized.

2.3. Virus isolates and cell culture

Virus isolates (Δ87, Δ184, Δ200, and Δ403, *wt*) were recovered from stored serum or lavage fluid using Marc-145 cell culture. The samples were selected from two pigs per group, the same animals as used for nsp2 sequencing as reported previously (Faaberg et al., 2010), for a total of 10 viral isolates. Additionally, viruses were isolated during the current swine study described above from serum and lavage fluid from two pigs per group for the repeat inoculation of *wt* (*wt*R) and for the pig passed large deletion virus (Δ403P), for an additional 4 viral isolates. The animals reinfected with large deletion virus cell supernatant fluid had no discernable viral titer, so the remaining sample for genome sequencing was selected based on the fact that nested RT-PCR confirmed the presence of viral RNA (Δ403R, Fig. 3). The 15 isolates were designated as the virus name (e.g., Δ87) followed by animal 1 or 2 (e.g., Δ87-1 and Δ87-2). Most isolates were generated from the last virus isolation positive sample, which was taken at 35 days post-infection, except for the swine passaged virus (Δ403P) where the two isolation positive samples were harvested at 15 dpi. The PRRSV nested RT-PCR positive serum sample (Δ403R) virus was blindly passed on MARC-145 cells. The original isolates were amplified by passage in two T75 flasks of Marc-145 cells. Virions were harvested by freeze-and-thaw of cultured cells exhibiting cytopathic effect (CPE), and then purified twice on a sucrose cushion (0.5 M sucrose, 10 mM NaCl, 10 mM Tris of pH 7.5 and 1 mM EDTA) by ultracentrifugation at 24,000 rpm at 4 °C for 3–4 h using SW28 rotor. Virus pellets were suspended in 0.5 ml STE buffer (10 mM NaCl/10 mM Tris, pH 8.0/1 mM EDTA, pH 8.0). To summarize, virus isolates Δ87-1, Δ87-2, Δ184-1, Δ184-2, Δ200-1, Δ200-2, Δ403-1, Δ403-2, *wt*-1, and *wt*-2 were recovered from stored samples obtained from a prior swine study (Faaberg et al., 2010). The present study yielded five additional virus isolates that were identified as *wt*R-1, *wt*R-2, Δ403R, Δ403P-1, and Δ403P-2.

2.4. Viral RNA extraction

Residual DNA contamination in the sucrose cushion purified virus suspension was eliminated by addition of 10 μl RQ1 DNase I (Promega) and incubated at room temperature for 10 min. RNase-In (25 μl, Ambion) and 50 μl of 0.1 M dithiothreitol (DTT) were added. Virus particles in suspension were then lysed by adding 0.5 ml of 2× LES buffer (0.2 M LiCl/10 mM EDTA/2.0% SDS) and 10 μl of proteinase K (20 mg/ml) and incubated at 56 °C for 30 min. The released viral RNA was extracted by adding equal volume of 0.1 M citrate buffer saturated phenol (pH 4.3) and half a volume of 24:1 chloroform:isoamyl alcohol followed by vortexing and incubation at 65 °C for 2 min, then cooled on ice for 1 min. The aqueous phase was separated by centrifugation for 3–5 min and then transferred to a new tube. Phenol extraction was repeated twice with heating, and then without heating. The aqueous phase containing viral RNA was extracted three times by an equal volume of 24:1 chlo-

roform:isoamyl alcohol and centrifugation. Viral RNA in the final aqueous phase was precipitated by addition of an equal volume of 7.5 M ammonium acetate (NH_4OAC) and two volumes of ethanol, followed by incubation at −70 °C for 30 min. Precipitated viral RNA was collected by centrifugation and the precipitate was washed once with 70% ethanol. The RNA pellet was collected by centrifugation and dried under vacuum for 5–10 min, and then resuspended in 50 μl of 10 mM Tris–HCl (pH 7.5). Purified RNA was quantified using a NanoDrop spectrophotometer and by PRRSV quantitative RT-PCR (qRT-PCR). Samples were also at times quantified by Quant-iT™ RiboGreen® RNA Kit (Invitrogen) and on an Agilent 2100 Bioanalyzer using the RNA 600 Pico chip.

2.5. Library preparation

The preparation of the libraries for sequencing followed the protocols, with modifications, used in other publications (Potgieter et al., 2009; Ramakrishnan et al., 2009; Roossinck et al., 2010). Purified viral RNA (200 ng) was fragmented by adding 2.5 μl of fragmentation buffer (200 mM Tris–acetate, 500 mM potassium acetate, 157.5 mM magnesium acetate [pH 8.1]) and incubation at 82 °C for 2 min. RNA samples were immediately transferred to ice and mixed with 10 mM Tris–HCl (pH 7.5) to bring the volume to 50 μl. The RNA was further purified by RNAClean using the manufacturer's recommendations (Agencourt) and then eluted in 9.5 μl of 10 mM Tris–HCl (pH 7.5). Successful fragmentation of the RNA was verified using the 2100 Bioanalyzer as described above. Synthesis of single stranded cDNA (sscDNA) from the fragmented RNA was achieved with a 5′ phosphorylated random primer (5′-phosphate-NNNNNNN-3′). The RNA samples were individually combined with 20 μl of 500 μM of reverse transcription primer (5′-phosphate-NNNNNNN-3′) and incubated at 70 °C for 10 min, and then cooled on ice. Reverse transcription reactions were assembled by adding 4 μl of first strand synthesis buffer, 1 μl of dNTP, 2 μl of DTT, 1 μl of superscript III enzyme (Invitrogen), and 0.5 μl of SUPERase-In RNase inhibitor (Ambion).

2.6. Adaptor ligation

Single stranded cDNA adaptors, both 5′ (sscDNA Adaptor A) and 3′ (sscDNA Adaptor B) were first prepared to allow directional ligation to the 5′-end and 3′-end of the sscDNA, respectively. A solution of 75 μl Oligo A (5′-GCCTCCCTCGCGCCATCAGACGAGTGCGT-3′; 200 μM) and 90 μl Oligo A′ (3′-dideoxyCGGAACGGTCGGGCGAGTCTGCTCACGCA-phosphate-5′; 200 μM), 3 μl 1 M Tris–Cl (pH 7.5) and 132 μl water was prepared in a thin-walled thermocycling tube, vortexed and spun. Another solution, 144 μl Oligo B (5′-biotin-GCCTTGCCAGCCCGCTCAGACGAGTGCGTNNNNNN-phosphate-3′; 500 μM) and 120 μl Oligo B′ (3′-dideoxyCGGAACGGTCGGGCGAGTCTGCTCACGCA-phosphate-5′; 500 μM), 3 μl 1 M Tris–Cl (pH 7.5) and 33 μl water was similarly prepared. The two sets of oligomers were then annealed to form double stranded DNA under the following thermocycling conditions: 5 min at 80 °C, 7 min at 65 °C, then 7 min at each successive 5 °C reduction in temperature until 25 °C, and then held at 4 °C. The resulting products, Adaptors A and B, were then vortexed, spun, aliquoted and stored at −70 °C. The unique sequences incorporated in Adaptor A and B facilitated the binding of the viral cDNA to the beads and clonal amplification of the cDNA attached to the beads. The Multiplex Identifier Sequences (MID; MID-1 highlighted in light grey above) in adaptors A and B were used to separate the samples post-sequencing in preparation for sequence assembly. Five MIDs were used. MID-1 is shown above, MID-2 was ACGCTCGACA, MID-3 was AGACGCACTC, MID-4 was AGCACTGTAG and MID-5 was ATCAGACACG. A key, TCAG, included in the oligomers and highlighted above in dark grey was used by the 454 sequencing software for recognizing library reads. The ligations were each performed in a total volume of 30 μl of 1× Quick Ligase Buffer (New England Biolabs) containing 12 μl of synthesized sscDNA, 1.67 μM of adaptor A and 6.67 μM adaptor B, and 200 units of Quick T4 ligase (New England Biolabs) at 37 °C for 2 h. The sscDNA ligated to the adaptors were each mixed with 0.5% Sera-Mag 30 beads (Seradyn/Thermo) in Binding and Wash Buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 M NaCl) and mixing for 10 min at room temperature. The unbound material was eliminated by washing with 0.5× Binding and Wash Buffer. The mixture was further washed twice with Bead Wash Buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 300 mM NaCl, 0.1% Tween-20) and then the ligated products were eluted from the beads with two rounds of Bead Elution Buffer (25 mM NaOH/1 mM EDTA pH 8.0, 0.1% Tween-20). The final sscDNA was purified twice by RNAClean and then dissolved in 12 μl of 10 mM Tris–HCl (pH 7.5). The adaptor-ligated sscDNA was evaluated on an Agilent 2100 Bioanalyzer using the RNA 600 Pico chip and quantified with a Quant-iT Ribogreen assay kit (Invitrogen) on a Synergy HT instrument (Bio-Tek) and quantitative RT-PCR.

2.7. Emulsion PCR and assembly of contiguous sequences

Four separate pools were created, each containing a mixture of samples labeled with the five unique adaptor-ligated viral sscDNAs (1×10^8 molecules of each sscDNA). The sscDNA pools were then diluted to a working concentration of 2×10^5 copies/μl and added in the appropriate ratio to primer-coated sequencing beads for Roche's GS-FLX standard chemistry emulsion-based PCR (emPCR). Prepared beads were loaded (up to 125,000 MID labeled beads/region) into four regions of an eight region GS-FLX standard chemistry pico-titer plate according to manufacturer's recommendations and sequenced using the GS-FLX LR 70 standard chemistry (Margulies et al., 2005). After completion of the sequencing run, each of the four pools had the reads for their respective five samples demultiplexed using the Roche sfffile program and the MID identifiers for each sample. The reads for each demultiplexed sample were then assembled into one or more contiguous sequences using GS Reference Mapper version 2.0 (454 Life Sciences) and PRRSV VR-2332 V7 sequence (GenBank DQ176021).

2.8. qRT-PCR

qRT-PCR, as previously described (Faaberg et al., 2010), was used to access the quality of the RNA and sscDNA. A Ct value of less than 20 cycles was found to yield the best pyrosequencing data.

2.9. RT-PCR

In order to confirm deletion size and ensure accidental contamination with full-length virus did not occur, viral RNA from sera containing virus from all animals per group (last virus isolation positive sample or d35 sera for Δ403R infected pigs) was extracted and subjected to differential analysis using One-Step RT-PCR (Qiagen), as described previously but with modification (Faaberg et al., 2010). In order to detect the virus used to inoculate the animals, 4 μl viral RNA was used from animals infected with Δ403R and Δ403P in the RT-PCR reaction in combination with primer pair 5′-2107 (5′-GACCTGTACCTCCGTGGTG-3′) and 3′-4300 (5′-CTGGGCGACCACAGTCCTA-3′) (1929 bp product). Nested PCR was completed using 1:100 dilution of first round product and primer pair 5′-2167 (5′-CGCCCGCCACGCGTAATCGACA-3′) and 3′-3846 (5′-TTGGTCAAAGAGCCTTTCAGCTTTT-3′) (492 bp). If *wt*R virus were present, a 1704 bp nested product would be seen. Viral RNA was similarly used in the RT-PCR assay from animals infected with *wt*R

virus with primer pair 5′-2392 (5′-CTAACCGCCGTGCTCTCCAAGT) and 3′-3846 (1479 bp product). Nested PCR was completed using primer pair 5′-2811 (5′-CCCACCTGAGCCGGCAACACCT) and 3′-3348 (5′-GCGTAGCAGGGTCATCAAGCTTAGTC) (563 bp product). If infected with Δ403 instead, no product would be seen in either step of the nested assay.

Standard RT-PCR was used to fill sequence assembly gaps that remained after reference mapped assembly of the pyrosequencing data. Genomic RNA of the virus isolates was used as templates in an individual RT-PCR reaction with primers flanking the gap region (primers available on request). The RT-PCR products were directly sequenced by the Sanger method (Sanger et al., 1977).

2.10. Viral RNA end amplification and sequencing by RACE

5′- and 3′-RACE (rapid amplification of cDNA ends; Takara Bio Inc.) were used to amplify the genome termini sequences of isolates. A 5′-end phosphorylated reverse primer (nt 862–848; 5′-GCCGGTTCGCAATCA) was utilized to copy the unknown 5′-end and the product was then circularized by the use of T4 RNA Ligase. A nested set of primers (5′-GCCGTTTTCGCCAATTCCCT/5′-GGCGGGGGAGCACTCAACA and then 5′-TTTTGCCCCTTTGAGTGTGCT/5′-TAGCACCCCGAGCTCAGAAAC) was used to amplify a DNA product that included the 5′-end of the genome. Similarly, a forward primer to cover the 3′-end VR-2332 genome (5′-CCAGAGGCAAGGGACCGGGA, nt 14825–14844] and the Oligo-dT-3 sites Adaptor Primer that contains restriction sites of BamH I, Kpn I and Xba I within its sequence from the 3′RACE kit for downstream cloning was utilized for 3′-end RACE. The amplified 5′- and 3′-end sequences were cloned into the pGEM-T vector (Promega) and at least two clones of amplified product were used to determine the 5′- and 3′-end sequences.

2.11. Sequence analysis

The whole genome sequences of all isolates were completed using Geneious software version 5.0.4 (Biomatters Limited), VR-2332 V7 sequence, the contigs from the assembly of pyrosequencing data, and the small RT-PCR products. In the case of detected small nucleotide polymorphisms (SNPs), the reported sequence represented the majority nucleotides.

3. Results

3.1. Clinical study

In order to further examine the growth kinetics and stability of the large deletion virus (Δ403), we performed an additional swine study as outlined in Section 2. We intramuscularly inoculated into swine two cell culture rescued recombinant viruses (*wt* and Δ403; 1×10^4 TCID each) to be used as experimental repeat controls, and one pig-passaged virus [4 ml Δ403-2 infected lung lavage sample positive for viral RNA (Faaberg et al., 2010)]. The results showed that infection with *wt* (no marked clinical signs) produced the highest LNW/BW ratios, statistically different ($P < 0.05$) from the results for infection with both large deletion virus groups (Fig. 2). To ensure that the animals were infected by the correct strain, RT-PCR and nested PCR were completed on late time-point samples, as previously done (Faaberg et al., 2010). The nested assay was able to detect a 492 bp product in only one animal from Δ403 repeat infection, but in all animals infected with Δ403 that had been previously passed in pigs (Fig. 3). The lack of a positive result for 5 of 6 ⊗403R animals was due primarily to the fact that an ELISA for recombinant PRRSV antigens showed a lack of seroconversion in 4 of them (PRRS 2XR, IDEXX laboratories; data not shown) and thus revealed that

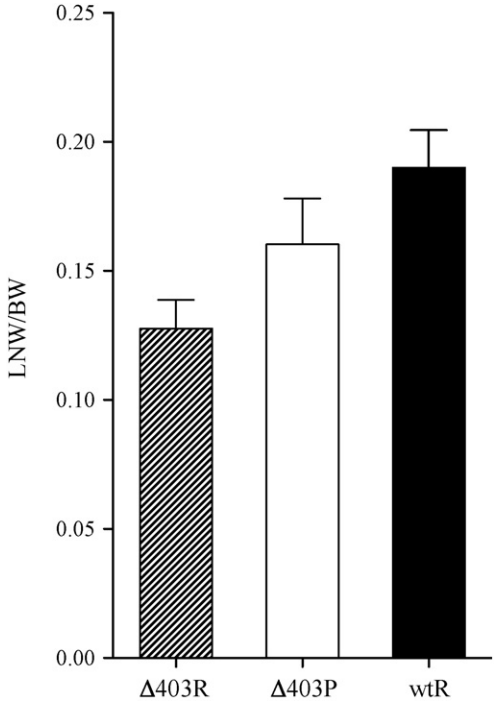

Fig. 2. The ratio of tracheobronchial lymph node weights to body weights (LNW/BW) for each animal was calculated and then averaged for each group (R = repeated; P = pig passaged). Bars with the standard error of the means indicated represent average ratios for each group.

the virus did not replicate in these swine. One additional ⊗403R animal did seroconvert, but the viral RNA present was not sufficient to be detected in the nested assay. A product of 1702 bp would have been derived if the animals had been contaminated with *wt*R virus. Animals infected with *wt*R, using alternative RT-PCR primers, had the expected nested RT-PCR product size of 563 bp.

3.2. Viral growth

Viral isolation and subsequent titer analyses (Fig. 4) demonstrated that swine reinoculation of recombinant *wt* virus resulted in moderate viremia for 21 days, whereas repeat inoculation of the large deletion mutant did not result in apparent viremia, in agreement with the prior study (Faaberg et al., 2010). However, inoculation of qRT-PCR viral RNA positive lung lavage (Δ403P) produced positive serum isolation results, signifying adaption of the large deletion virus. The deletion virus growth kinetics were less vigorous than that of the *wt* virus at all time points tested, except for 15 dpi (Fig. 4A), which suggested a gain of additional deletion virus fitness with time. No virus was detected in the serum after 21 days, and only *wt* recombinant virus was isolated from the lavage fluid at day 35 (Fig. 4B).

3.3. Nucleotide sequence analysis

Previous work had demonstrated that the Δ87, Δ184, Δ200, and Δ403 nsp2 deletion mutants were genetically stable in the region coding for the large replicase protein (Faaberg et al., 2010). In order to assess whether other areas of their genomes had remained stable or had compensated genetically for the nsp2 deletion after growth in swine, massively parallel pyrosequencing technology was employed using 454 technology (Margulies et al., 2005). The viral isolates obtained for mutants Δ87-1, Δ87-2, Δ184-1, Δ184-2, Δ200-1, Δ200-2, Δ403-1, Δ403-2 and *wt*-1 and *wt*-2, corresponding to the same 35 dpi swine serum used for prior nsp2 sequence

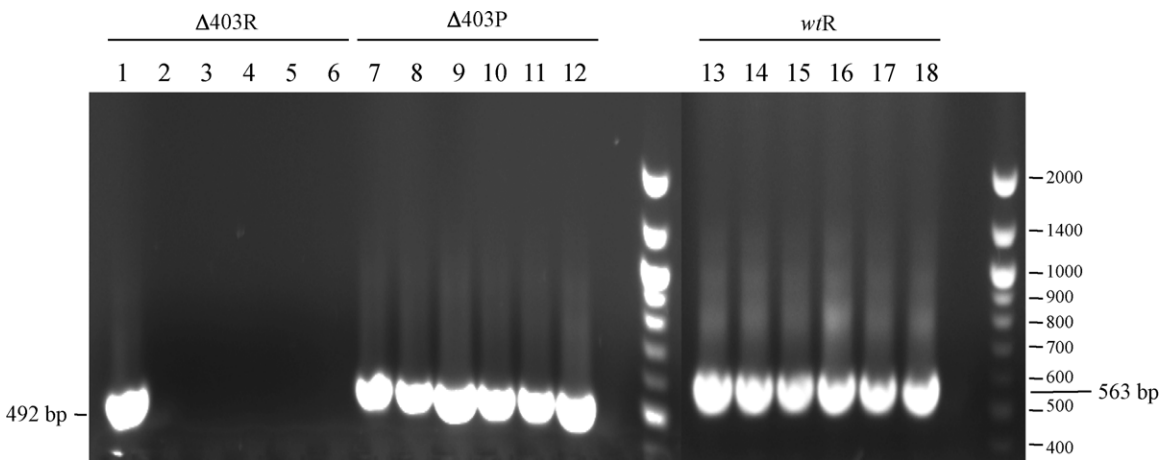

Fig. 3. Nested RT-PCR detection of PRRSV in day 35 serum to confirm construct originally used to infect 6 pigs/group. Lanes 1–6: Δ403R (R = repeated; 492 bp product); Lanes 7–12: Δ403P (P = pig passaged; 492 bp product); Lanes 13–18: *wt*R (563 bp product). No *wt* virus was detected (1704 bp product) in Lanes 1–12.

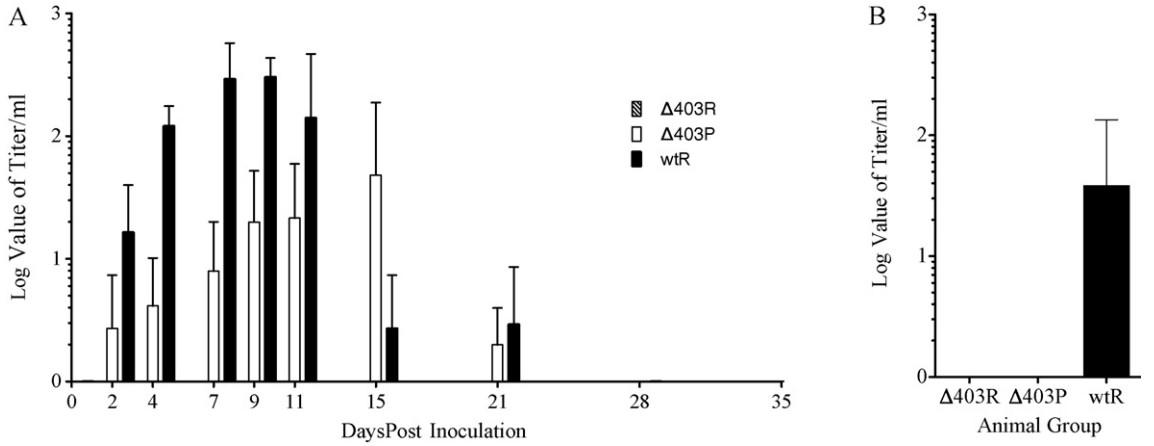

Fig. 4. Virus isolation. Each swine sample on each day was analyzed for growth on Marc-145 cells and titrated. The average titer and the standard error of the mean were plotted for each group on each day. (A) Serum samples. (B) Lavage samples.

analysis (Faaberg et al., 2010), were amplified and purified using two successive sucrose density cushions as described in Section 2. Virus isolates from the present clinical study, derived from two animals from the Δ403P and *wt*R groups and one animal from the Δ403R group, were amplified and purified. Near to full genome sequences of most isolates were achieved by a single sequencing run on a pico-titer plate, in which thousands of reads of individual short (~300 base) sequences were acquired. The reads were sorted out by MID sequence and suitable reads of each MID-labeled genome were selected and assembled into one or several contigs. The reads of 5 PRRSV isolates were each further assembled into a single contig/isolate, while reads of 8 isolates were assembled into 2–7 contigs, leaving 1–6 gaps of mostly 100–200 bases between the contigs (Table 1). The contigs for these 13 isolates covered 98.3–99.9% of the virus genome, except Δ403P-1 that covered 95.2%. There were only two isolates (*wt*-1 and Δ403P-2) for

Table 1
Pyrosequencing reveals near full-length genomes were acquired except for 2 isolates.

Genome	Filtered reads	% filtered reads	# Contigs	Bases covered	% coverage
Δ87-1	5100	85.3	1	15092	99.6
Δ87-2	1912	53.2	3	15058	99.4
Δ184-1	2668	74.1	5	14680	98.8
Δ184-2	3908	87.9	1	14613	98.3
Δ200-1	2073	72.3	1	14673	99.1
Δ200-2	1710	62.6	2	14560	98.3
Δ403-1	2108	44.9	1	14179	99.8
Δ403-2	3845	83	4	14081	99.1
Δ403R-1	2500	78.7	2	14018	98.7
Δ403P-1	524	9.9	7	13521	95.2
Δ403P-2	178	8.7	16	9148	64.4
wt-1	528	29.2	11	14486	94.0
wt-2	3018	67.5	3	15321	99.4
*wt*R-1	1568	39.2	5	15402	99.9
*wt*R-2	1177	68.5	1	15162	98.4

Table 2
Summary of number of nucleotide and amino acid changes occurring after swine passage.

	Δ87-1	Δ87-2	Δ184-1	Δ184-2	Δ200-1	Δ200-2	Δ403-1	Δ403-2	Δ403R	Δ403P-1	Δ403P-2	*wt*-1	*wt*-2	*wt*R-1	*wt*R-2
Nucleotide															
5′UTR	–	–	–	–	–	–	1	1	1	2	1	–	–	–	
ORF1ab	5	6	20	15	17	12	12	13	17	13	18	7	9	7	8
ORFs 2–7	–	–	–	1	1	1	–	3	2	1	4	2	–	3	2
3′UTR	–	–	2	–	–	–	2	1	–	–	–	–	–	1	–
Amino acid															
nsp1α	1	–	1	–	1	2	2	–	2	1	2	2	1	–	1
nsp1β	–	–	1	2	–	–	1	1	1	1	1	–	–	–	–
nsp2	–	–	1	4	5	6	4	4	2	3	6	1	2	1	1
nsp3	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–
nsp4	–	2	1	–	1	–	–	1	–	–	–	–	–	–	–
nsp5	–	–	1	–	–	–	1	2	1	3	2	–	1	–	–
nsp6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
nsp7α	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
nsp7β	–	–	–	1	–	1	1	–	–	–	–	–	–	–	–
nsp8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
nsp9	–	–	1	–	1	–	1	1	1	1	1	–	–	–	–
nsp10	1	–	1	2	–	–	–	–	–	1	1	1	1	1	1
nsp11	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1
nsp12	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–
E	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
GP2	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–
GP3	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–
GP4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
GP5	–	–	–	–	–	–	–	–	–	–	1	1	–	1	1
M	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–
N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

which 11 and 16 contigs were assembled, leaving 10–15 gaps and genome coverage of 94% and 64.4%, respectively (Table 1). Overall, these results demonstrated that 454 pyrosequencing technology is a powerful tool for medium and large virus genomic research when full-length genomic sequence is required from many study animals.

3.4. Genomic ends and gap sequences

Analysis of the 454 sequencing protocol and downstream contig assembly revealed that pyrosequencing technology was not ideally effective in generating 5′- or 3′-genomic ends. Therefore, RACE was performed for all isolates, and two separate clones containing the 5′ and/or 3′ end nucleotides were sequenced. RT-PCR was performed with gene specific primers, and PCR products were sequenced in both directions to fill missing gaps.

3.5. Sequence alignment and analysis

Assembled genome sequence alignments showed all of the nsp2 deletions were well maintained during both passages in vivo, which confirmed previous RT-PCR sequencing results. There were no sequence deletions or insertions elsewhere in the genome of *wt* virus and the nsp2 deletion mutants, which further demonstrated the fact that deletion size did not affect the in vivo relative genomic stability. *Wt* viruses (*wt* and *wt*R) and the small deletion mutants (Δ87) showed less nucleotide change when compared to the larger deletion mutants (Δ184, Δ200 and Δ403) (Table 2 and Fig. 5). Five to 23 nucleotide substitutions were observed throughout the genome in all *wt* and nsp2 deletion mutants, the majority residing in the nsp2 region (Table 2 and Fig. 5). There was, however, the tendency for silent nucleotide substitutions or semi-conservative amino acid changes in *wt* and the Δ87 mutants, while the nucleotide substitutions in the Δ200 and Δ403 deletion mutants resulted in both conservative and nonconservative amino acid changes (Table 3). Most of the nucleotide substitutions and amino acid changes occurred in the nonstructural protein coding regions of all isolates (Table 3). Lastly, there were very few changes that occurred in more than 1 isolate of a particular virus. All viruses had the G→E mutation at aa 3677 of the replicase protein (nsp11). The infectious clone of strain VR-2332 was previously shown to have a G residue at this site, identical to the VR-2332 vaccine strain Ingelvac® MLV (Han et al., 2007). This E residue was also found conserved across all Type 1 and 2*wt* viruses (data not shown), and most likely represents a critical residue for optimum nsp11 protein function. The most intriguing changes occurred in all Δ403 mutant viruses. Specifically, the A→G nucleotide mutation in the 5′ leader and the R285C (nsp1β; nonconservative), L2150I (nsp5; conservative), and T2752N (nsp9; semiconservative) aa mutations in ORF1. Each of these sites was altered from residues conserved in all *wt* Type 2 viruses, although they have never been shown to be associated with a specific viral function.

4. Discussion

The animal study revealed similar results to the initial trial (Faaberg et al., 2010) in that inoculation with the large nsp2 deletion mutant (Δ403R) produced no virus isolation titer after inoculation of swine and only one animal showed signs of viral replication on day 35, evidenced by nested RT-PCR and regional sequencing. Importantly, we found that an additional swine passage of this mutant virus (Δ403P) resulted in increased replication fitness, yet the mutant virus showed a delay in reaching its highest titer when compared to *wt* infection (day 15 vs. day 7–9) (Fig. 4A). No virus was recovered in the lung lavage fluid of the Δ403R and Δ403P infected animals, indicating that the increased replication rate of Δ403P did not result in retention of virus in that organ (Fig. 4B). However, the LNW/BW results indicated that an additional passage in swine of this large nsp2 deletion virus increased the lymph node hyperplasia (Fig. 2). We conclude that nsp2 can be deleted by at least 403 aa in HV2 and still successfully replicate in swine, although with reduced kinetics. Whether additional passage would improve the virus replication further remains to be determined.

Fifteen full-length sequences of viruses isolated from swine samples after in vitro passage were generated by 454 pyrosequencing technology. Foremost, this study revealed that the four

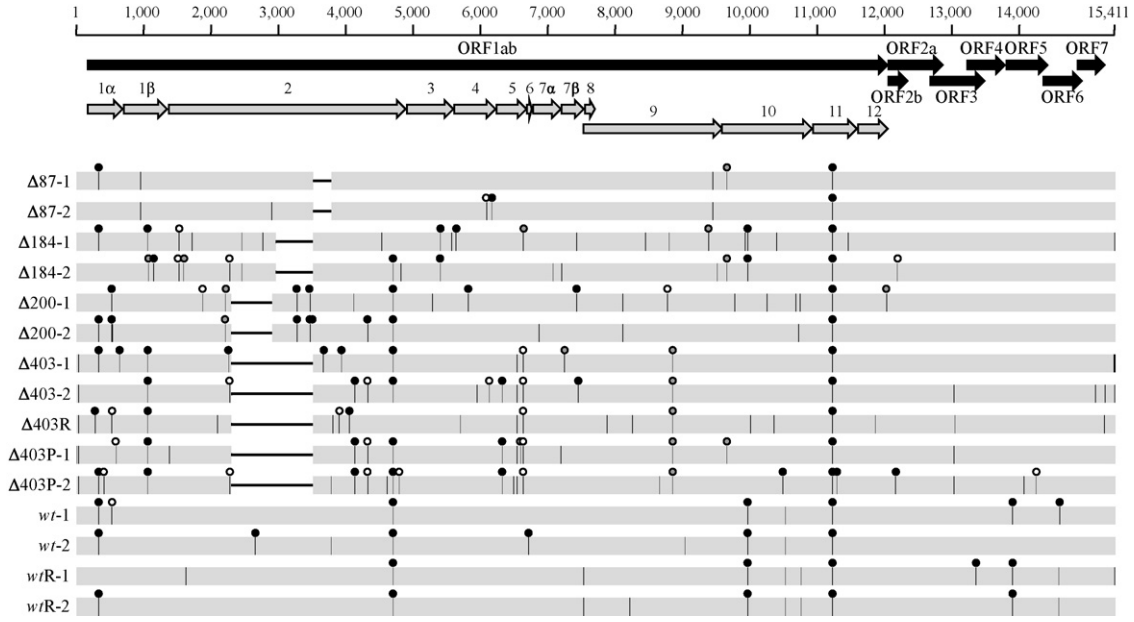

Fig. 5. Schematic representation of the individual ORFs and nsps encompassing the nucleotide changes that occurred from parent clone pVRV7 sequence throughout the genome for each isolate after growth in individual swine. Nucleotide alterations that resulted in a nonconservative amino acid change are depicted by a closed circle (●), a semi-conservative change by a grey circle (⬤) and a conservative change by an open circle (○).

Table 3
Amino acid mutations seen in both genomes of sequenced isolates after PRRSV rV7 replication in vivo. Amino acid mutation types are indicated as follows: conservative, not shaded; semi-conservative changes, light grey; nonconservative mutations, dark grey. Transition (Ts) and transversion (Tv) sites are also indicated.

Region	Nucleotide substitution	Type of substitution	Δ87	Δ184	Δ200	Δ403	Δ403R	Δ403P	wt	wtR
5′ leader	A39G	Ts				A → G	A → G	A → G		
nsp1α	C526A	Tv			P → T					
	C960U	Ts	Silent							
nsp1β	C1051U	Ts				R → C	R → C	R → C		
nsp2	C1526U	Ts		A fl V						
	C2212U	Ts			L → F					
	U2463C	Ts		Silent						
	G3278A	Ts			G → E					
	A3463U	Tv			R → W					
	G4125U	Tv						Silent		
	U4322C	Ts						V → A		
	A4702G	Ts			K → E	K → E		K → E	K → E	K → E
nsp3	U5402C	Ts		M → T						
nsp5	A6316G	Ts						S → G		
	A6546C	Tv				Silent	Silent	Silent		
	C6637A	Tv				L → I	L → I	L → I		
nsp7	C7530U	Ts								Silent
nsp9	U8108A	Tv			Silent					
	C8842A	Tv				T → N	T → N	T → N		
	U9437C	Ts	Silent							
nsp10	G9958A	Ts		G → E					G → E	G → E
	A10520U	Tv							Silent	Silent
	C10754U	Ts								Silent
nsp11	G11221A	Ts	G → E	G → E	G → E	G → E	G → E	G → E	G → E	G → E
GP3	U13026C	Ts						Silent		
GP5	G13887A	Ts								D → N
GP6	U14575C	Ts								Silent

engineered deletion mutant viruses, with 87-403 aa removed from the second hypervariable region of nsp2, were stable in swine for at least one passage, and that the largest deletion mutant was stable for two passages. The sequencing data showed that the numbers of quasispecies were minor and occurred at only a few single nucleotide sites per sequencing run (data not shown). Furthermore, the 454 sequencing yielded only one major genome and, in examining the raw data, did not show evidence of further deletions in any region of the mutant viruses, whereas sections of two different quasispecies were seen in another Type 2 virus sequenced in the same experiment (data not shown). There is the minor chance that viruses with additional deletions elsewhere in the PRRSV genome were not propagated during isolation and propagation on Marc-145 cells. The single virus species detected in each case suggested that

any additional virus variants that may have arisen during replication in swine were in low abundance or were defective.

When the full-length genomes were compared to each other and to parental rVR-2332, very few changes were noted, and most were viral isolate specific. The nsp2 mutant with smallest deletion, Δ87, showed the least divergence from the respective input virus compared to all other deletion viruses tested, and also to *wt* virus (for unknown reasons) (Table 2 and Fig. 5). However, when deleting a larger section of nsp2 hypervariable region 2, several more mutations were seen, as noted above. However, no mutation was seen that corresponded to the increased ability to replicate in swine upon an additional passage. The data from 454 sequence analysis clearly showed that large nsp2 deletions, although stable for at least one passage in swine, led to only a few more pronounced single amino acid changes elsewhere in the viral genome. We concluded that the nucleotide and/or amino acid mutations that did occur resulted in a more replication fit virus, or that a viral quasispecies enabling the enhanced viral growth kinetics seen was present but not detectable with 454 sequencing technology.

We conclude that PRRSV strain VR-2332 remains relatively stable even after substantial deletions in the large hypervariable region of nsp2. Further work will examine the changes in the host immune response after inoculation of these nsp2 deletion mutants into a larger group of swine.

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